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A pathogenic role of IL- 17 at the early stage of corneal allograft rejection

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ABSTRACT

Purpose: Th17, recently identified as a new subset of effector Th cells, has been shown to be involved in microbe infection and autoimmunity. However, the role of these cells in organ allograft rejection remains largely unknown. In this study, we investigate whether Th17 cells participate in allogeneic corneal rejection in a mouse model.

Methods: Donor cornea (C57BL/6) was transplanted into orthotopic graft bed of Balb/c recipients. At different time points after keratoplasty, the expression of Th17 and Th1- related cytokines in draining cervical lymph nodes (LN) and grafted cornea was examined by flow cytometry and quantitative RT- PCR, respectively. Furthermore, IL- 17−/− Balb/c mice were used to determine the effects of Th17 cells on allogeneic cornea survival. Finally, the profiles of Th1 and proinflammatory cytokines in IL- 17−/− recipients after transplantation were examined.

Results: Th17 expression was enhanced significantly in inflamed transplants and draining lymph nodes at the early stage of allocorneal rejection, while upregulation of Th1 producing IFN- γ was seen in the late phase. Upon activation by allogeneic accessory cells, responder cells in draining LN from transplanted recipients secreted high levels of IL- 6, TGF- β and IL- 21 compared to controls, which may drive naive T cells to differentiate into Th17 cells. Importantly, IL- 17 deficiency led to the delayed development of allogeneic rejection, but did not affect the overall survival time of transplants. This effect correlated with restrained Th1 polarization and decreased production of proinflammatory cytokines.

Conclusion: Th17 cells play a disease-promoting role at the early stage of corneal allograft rejection.

1. Introduction

CD4+ T helper (Th) cells are important mediators in immune responses, acting to coordinate the other cellular components of the immune system. Recently, three Th subsets have been characterized: Th1, Th2 and Th17 cells [1]. Th1 cells, with the characteristics of secreting IFN- γ and expressing T- bet as well as STAT4, are pivotal for the clearance of intracellular bacteria [2]. Th2 cells play a critical role in humoral immunity by producing IL- 4, IL- 5 and IL- 13, whose commitment is controlled by master regulator GATA- 3 [2]. As to Th17 cells, they produce large amounts of IL- 17, IL- 17F, IL- 21 and IL- 22, contributing to mucosal immunity [3,4]. When exposed to TGF- β, IL- 6 and IL- 21, naive T cells differentiate into Th17 cells under control of housekeeper gene RORγt [5]. In addition, albeit dispensable for Th17 commitment, IL- 23 is required for Th17 expansion and phenotype persistence [6,7]. Apart from helper T subsets, regulatory T cells (Treg) is another T cell lineage under the control of master gene Foxp3 [8], which is involved in preventing excessive immune response to self or non- self antigens [9].

The importance of Th17 cells in the control or clearance of various pathogens has been identified in several models of acute infection [10–12]. These cells are also key pathogenic elements in several autoimmune diseases, which were previously assumed to be dominated by Th1 responses [13–15]. Interestingly, Th17 cells have been shown recently to be implicated in allograft rejection of solid organs [16–20]. Vanaudenaerde et al. observed that, in patients with acute rejection of lung transplantation, IL- 17 mRNA and protein levels were increased in bronchoalveolar lavages [17]. Moreover, Th17- mediated immunity to collagen type V ultimately caused progressive airway obliteration in the transplanted lung [18]. The disease- promoting role of Th17 in cardiac allograft rejection was also confirmed, especially in the absence of Th1 response [19,20]. However, Th17 seems not to do so invariably in transplantation. In graft- versus- host disease (GVHD), although some reports demonstrated pathogenicity of Th17 in inducing GVHD [21,22], Kappel et al. found that IL- 17 deficiency just contributed to delayed GVHD development, but overall mortality was unaffected [23]. More importantly, a recent study showed that the absence of donor Th17 cells resulted in enhanced Th1 differentiation and exacerbated acute GVHD and administration of recombinant IL- 17 or neutralizing IFN- γ in the recipients given IL- 17−/− donor cells

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ameliorated the acute GVHD [24]. Thus, it is necessary to further elucidate the role of Th17 in allograft rejection.

Penetrating keratoplasty in normal eyes is emerging as the most common and successful form of solid tissue transplantation [25]. However, corneal grafts in high- risk (i.e., immune- privilege- abrogated) eyes continue to carry a poor prognosis. Even intensive systemic immunosuppressive therapy is often of no avail [26]. Therefore, a better understanding of rejection mechanism is necessary for the development of new immunomodulatory strategies in patients who undergo keratoplasty. Although several factors recently have been confirmed to be involved in acute corneal allograft rejection, to our knowledge, the role of Th17 in this kind of solid organ transplantation is still elusive. In this study, we examine whether Th17 cells are generated during acute corneal allograft rejection and up and down- stream regulators for Th17 development. In addition, we used IL- 17-deficient mice to assess whether IL- 17 is required for acute corneal rejection.

2. Objectives

The objectives of this work were the following:

- To determine the dynamics of Th17 and Th1 expressions in draining LN as well as the inflamed cornea after allogeneic corneal transplantation
- To investigate the effects of IL- 17 deficiency on cornea allograft rejection
- To analyze whether IL- 17 deficiency affects the development of Th1 and proinflammatory cytokines.

3. Materials and methods

3.1. Mice and anesthesia

Male Balb/c (H-2^d) and C57 BL/6 (H-2^b) mice aged 8 to 10 weeks were purchased from Experimental Animal Center of Shanghai. IFN- γ−/− Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). IL- 17−/− Balb/c mice were supplied by Institute of Experimental Animal, Chinese Academy of Medical Sciences (Beijing, China). Before all surgical procedures, each animal was deeply anesthetized by intraperitoneal injection of 3 to 4 mg of ketamine and 0.1 mg of xylazine. Care, use and treatment of mice in this study were in strict agreement with the guidelines in the care and use of laboratory animals set forth by Zhejiang University.

3.2. Orthotopic corneal transplantation and assessment of graft survival

Balb/c mice ($n=54$) were used as recipients of syngeneic (Balb/c, $n=10$), or major histocompatibility complex (MHC)- mismatched (C57BL/6, $n=42$) corneal transplants. The corneal transplantation technique was performed according to a standard operation. Mydriasis in the eyes of the recipients was achieved by local application of tropicamide phenylephrine eyedrops (Santen, Janpan) for three times at intervals of 10 minutes before the operation. Upon anesthetization, the donor button was excised with Vannas scissors and placed in Optisol corneal preservation solution. The recipient graft bed was prepared by excision of the equal size of the cornea. The donor button was then secured with eight to ten interrupted 11-0 silk sutures (Sharpoint, 100 Dennis Drive, PA, USA). To protect the transplant, a blepharorrhaphy was performed by means of two interrupted sutures (Prolene 10.0), which remained in place for 24 hours, antibiotic ointment was applied in the palpebral fissure. All grafted eyes were examined after 24 h; Transplant sutures were removed in all cases on day 7. Grafts were examined by slit lamp biomicroscopy twice a week. In some setting, grafted cornea survival was evaluated every day. At each timepoint, grafts were scored for opacification from 0 to 5+, according to a scoring system described elsewhere [27]. Grafts exhibiting an opacity score of 3+ or greater (moderate stromal opacity with only pupil margin visible) at 2 weeks or 2+ or greater (mild deep stromal opacity with pupil margin and iris vessels visible) after 3 weeks were considered rejected (immunologic failure).

3.3. Flow cytometry

Cells were restimulated with 50 ng/ml PMA (Sigma- Aldrich), 1 mg/ml ionomycin (Sigma- Aldrich), and GolgiPlug (Pharmingen) at 37 °C for 12 hours. Cells were first stained for CD4 antigen using FITC-labeled CD4 antibody (GK1.5, *e*Biosciences) then treated with Cytofix/Cytoperm (Pharmingen) according to the manufacturer's instructions. Intracellular staining was performed as follows: the permeabilized cells were stained with PE- labeled anti- murine IL- 17 (TC11- 18H10, Pharmingen), Foxp3 (FJK- 16S, Pharmingen), APC- labeled anti- murine IFN- γ (XMG1.2, Caltag Laboratory) or irrelevant isotypes (Pharmingen) on ice in the dark for 30 minutes. Data collection and analysis were performed on a FACS Calibur flow cytometry using CellQuest software (Becton Dickinson, USA).

3.4. Preparation of lymphocytes from graft recipients and coculture experiments

Draining cervical lymph nodes (LNs) and spleens were harvested from graft recipients and pressed through nylon mesh to produce a single-cell suspension. Lymphocytes from each recipient were separated in the subsequent experiments. Red blood cells were lysed by TE buffer (10 mM Tris– HCl and 0.1 mM EDTA) and washed twice. In cell culture experiments, T cells were purified to more than 95% by Thy1.2 microbeads according to the manufacturer's instructions (MACS; Miltenyi Biotec GmbH, Bergisch- Gladbach, Germany). In mixed lymphocyte reaction (MLR), irradiated splenocytes from donor naive mice served as allogeneic and syngeneic APCs, respectively. Single- cell suspension of splenocytes was prepared in AIM- V medium (Invitrogen Life Technologies, Grand Island, New York) containing 1% FBS. RBCs were removed as described above. The cells were then washed twice, irradiated at 2000 rads, and resuspended in FBS- free AIM- V medium. T cells (10^5) were stimulated by allogeneic or syngeneic APC (10^4) in 96- well plates for 48 hours. On the end of coculture, the supernatants were collected for subsequent experiments.

3.5. ELISA for cytokine production

After 48 h coculture, the supernatants were collected and examined for IL- 17, IL- 17F, IL- 22, IFN- γ, IL- 6, IL- 4, IL- 12, IL- 21, IL- 23, TNF- α, TGF- β production according to the instructions of manufacturers. IL- 17, IFN- γ, IL- 6, IL- 4 and IL- 12 antibody pairs for ELISA were purchased from BD Pharmingen (USA). IL- 23 and TNF- α Ready- Set- Go ELISA kits were from *e*Biosciences. IL- 17F, IL- 21, IL- 22 and TGF- β DuoSet ELISA kits were purchased from R&D systems (USA).

3.6. Quantitation of cytokine transcripts

Corneal tissues were homogenized in RNA STAT- 60 (1 ml/50 mg tissue, Tel- test) in a Polytron homogenizer. Following homogenization, RNA extraction, precipitation and wash was performed routinely. Quantitative RT- PCR was performed with the use of an ABI PRISM 7700 sequence- detection system with SuperScript One- Step RT- PCR System (Invitrogen). Predesigned primer/probe sets were from Applied Biosystems. Probe specific for GAPDH was used as internal controls. Each measurement of cytokines was normalized to expression of GAPDH (delta Ct). The inverse log of the delta- delta Ct was then calculated to give the fold change.

3.7. Statistical analysis

Log- rank test was used to compare survival time of grafts. Student's *t*- test was used to compare mean values. Values of $p<0.05$ were considered significant.